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JANUARY 15 TRIANNUAL REPORT FOR: EFFECTS OF LYOPHILIZATION ON METABOLIC INTEGRITY
OF RED BLOOD CELLS

Grant No.: N00014-94-1-0402

Performance Site: School of Pharmacy, University of Colorado Health Sciences Center

Principal Investigator: John F. Carpenter, Ph.D.

Progress reporting period: September 1, 1994 - December 31, 1994 (i.e., Quarter 3 for Year 1)

I. Subprojects listed in Proposal Milestones Chart

A. Subproject A. Evaluate metabolism of fresh cells

A.1. Evaluate glycolytic flux, metabolite levels and PFK kinetic properties

Status: Work in progress

Accomplishments:

1. The column needed for the HPLC assay of adenylates was changed to an aminopropyl column, when it was discovered that the old reverse phase column had to be cleaned extensively after every 4-5 biological samples were assayed. A new protocol for the aminopropyl column has been identified. With this protocol and column, we should be able to run at least 400-500 samples before having to clean the column. Thus, our overall efficiency will be greatly improved.

2. The HPLC assay for glycolytic intermediates has been improved to resolve two overlapping peaks for key metabolites. This approach, plus installing a larger sample loop, should eliminate the need for fractionating samples prior to running the assay. A separate column and protocol for measuring the triosephosphate glycolytic intermediates has been identified, which should allow for quantitation of these previously difficult to assess compounds.

A.2. Characterize enzyme binding to membrane

Status: Initiation of work delayed

B. Subproject B. Evaluate metabolism of lyophilized/rehydrated cells

B.1 and B.3 Metabolism immediately after rehydration and under simulated *in vivo* conditions.

Status: Work in progress

Accomplishments: We have successfully implemented the rehydration and wash procedure, which was developed by Dr. Spargo's group, for the lyophilized red cells. The cells survived subsequent transfer into our "metabolic assessment buffer", which is composed of: 50 mM Tris (pH 7.3 at 37°C), 125 mM NaCl, 50 mM glucose, 6% (wt/vol) hydroxyethyl starch and 25 ppm gentamicin. The cells had essentially the same rate of metabolic heat generation as control cells for the duration of the 18-hour 37°C experiment in the microcalorimeter. These results indicate that in this buffer system, which mimics major conditions (i.e., osmolality, oncotic pressure, pH, 37°C etc.) found in human plasma, that the lyophilized/rehydrated cells have a "normal" overall metabolic rate.

B.4 Evaluate metabolism after dried storage and rehydration

Status: Work in progress

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Approved for public release
Distribution Unlimited

Accomplishments: Ideally, the dried red cells should be able to withstand long-term exposure to room temperature and higher temperatures, which may be encountered during shipping and storage in regions of world where refrigeration is not available. Prior to initiating storage studies, it was important to determine the maximum storage temperatures that may be acceptable for the dried cells. For most dried biomaterials, it is generally considered that the highest storage temperature must be under the glass transition temperature, to avoid damage. Therefore, before initiating storage studies, we had to determine the glass transition temperature of the dried red cell preparations. Our (with Dr. Spargo) analyses with differential scanning calorimetry indicated that the glass transition temperature was about 60°C. This is excellent, since it means from a strictly physical standpoint that it should be possible to store the cells at normal ambient temperatures. Since Dr. Spargo's group plans to make alterations in the lyophilization process/formulation to improve acute recovery of red cells, we will have to repeat these studies to determine the glass transition temperature of the new samples, prior to starting storage stability studies.

C. Subproject C. Development of quality control testing of red cells

C.1. Assess utility of HPLC for ATP and 2,3DPG assays

Status: Work in progress

Accomplishments: no work since last report

C.2. Assess utility of calorimetry for determining overall metabolic integrity.

Status: Work in progress

Accomplishments: see section B above

II. New Subprojects not listed in Milestones Chart

D. Subproject D. Determination of conditions needed to maximize ATP and 2,3-DPG levels prior to lyophilization

Status: Work in progress

Accomplishments: no work since last report

E. Subproject E. Determination of metabolic integrity of red cells stored in standard blood banking solutions

Status: Work in progress

Accomplishments:

1. A new long-term (i.e., six weeks) study of the metabolic integrity of red cells stored in liquid at 4°C has been started. A full report of the data will be provided at the completion of the study (i.e., in the next Triannual report).

Subproject F. Infrared spectroscopic analysis of conformational integrity of hemoglobin in lyophilized red cells

Status: Work in progress

Accomplishments:

1. We have used infrared spectroscopy to evaluate the secondary structure

of hemoglobin in more samples of lyophilized and lyophilized/rehydrated red cells. The current results have quantitated the degree of change and agree with the earlier more qualitative results. The hemoglobin is unfolded in the dried solid, but refolds during rehydration. Many studies with purified hemoglobin (including some of our preliminary work) have indicated that the rate of formation of methemoglobin is greatly increased if the protein is unfolded. If the same relationship is true for hemoglobin in the dried red cells, then it may be necessary to dry the cells such that hemoglobin does not unfold. Even though the protein refolds during rehydration, the presence of unfolded hemoglobin in the dried cells may lead to excessive methemoglobin formation during storage. Therefore, it may be important to optimize the lyophilization process and formulation for retaining native hemoglobin in the dried solid.

2. In order to obtain this goal it is crucial to determine the separate effects of the freezing and drying steps of lyophilization on hemoglobin's conformation. This is because the alterations in the formulation needed to prevent damage at these two steps differ. Freezing protection derives from the bulk concentration of the stabilizer (i.e., the glucose), whereas protection during drying is dependent on the mass ratio of stabilizer:protein and the presence of an amorphous stabilizer. If the damage arises primarily during freezing, then it may be necessary to increase the bulk concentration of glucose to even higher levels than are currently used. Conversely, if unfolding only arises during the subsequent drying, it may be possible to minimize this damage by keeping the cells below the T_g' of the cellular cytosol, such that glucose remains amorphous during dehydration. These physical transitions would have to be determined with differential scanning calorimetric assessment of isolated red cells.

We have designed a modification of our freeze-drying reflectance accessory that should make it more amenable for studies of red cells. The custom components needed are being manufactured. We will begin work with red cells as soon as the new modified accessory is ready.

Product: A manuscript describing the quantitation of lyophilization-induced alterations in protein secondary structure has been accepted for publication in the *Journal of Pharmaceutical Sciences*. Copies of the preprint are enclosed.

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